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superroot, a Recessive Mutation in Arabidopsis, Confers Auxin Overproduction

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We have isolated seven allelic recessive Arabidopsis mutants, designated *superroot* (*sur1-1* to *sur1-7*), displaying several abnormalities reminiscent of auxin effects. These characteristics include small and epinastic cotyledons, an elongated hypocotyl in which the connection between the stele and cortical and epidermal cells disintegrates, the development of excess adventitious and lateral roots, a reduced number of leaves, and the absence of an inflorescence. When germinated in the dark, *sur1* mutants did not develop the apical hook characteristic of etiolated seedlings. We were able to phenocopy the Sur1$^-$ phenotype by supplying auxin to wild-type seedlings, to propagate *sur1* explants on phytohormone-deficient medium, and to regenerate shoots from these explants by the addition of cytokinins alone to the culture medium. Analysis by gas chromatography coupled to mass spectrometry indicated increased levels of both free and conjugated indole-3-acetic acid. *sur1* was crossed to the mutant *axr2* and the *altered-auxin response* mutant *ctr1*. The phenotype of both double mutants was additive. The *sur1* gene was mapped on chromosome 2 at 0.5 centimorgans from the gene encoding phytochrome B.

INTRODUCTION

A plethora of experiments investigating the physiological effects of exogenously applied phytohormones has shown that auxins participate in a wide variety of processes, such as cell expansion, apical dominance, vascular differentiation, lateral root development, phototropism, and root gravitropism (Davies, 1987). The development of molecular biology and gene transfer technology has made it possible to study the physiological effects of elevated endogenous auxin levels. The expression of bacterial auxin biosynthesis genes in plants results in the overproduction of both indole-3-acetic acid (IAA) and IAA conjugates (Klee et al., 1987; Sitbon et al., 1991, 1992, 1993; Romano et al., 1993). The expression of the bacterial IAA–lysine synthetase gene leads to the accumulation of IAA conjugates in plants but not necessarily to a comparable decrease in the level of free IAA (Romano et al., 1991; Spena et al., 1991). These studies strongly suggest that the level of free IAA in the plant is controlled by both de novo biosynthesis and conjugation (reviewed by Hobbie and Estelle, 1994).

The molecular mechanisms involved in auxin biosynthesis, sensitivity, and signal transduction are still poorly understood. Questions regarding which genes are involved in the biosynthesis of auxin, how the level of auxin is regulated in the plant, and what the basis is for the differential sensitivity of different tissues to auxin remain to be answered. The isolation of mutants with altered responses to auxin and the cloning of the corresponding genes promise to be a valuable strategy with which to address these questions. A number of Arabidopsis mutants resistant to growth-inhibiting concentrations of auxin have been isolated (for a review, see Hobbie and Estelle, 1994). In addition to being resistant to auxin, several of these mutants, namely, the *altered-auxin response* (*axr1* and *axr2*) and the *auxin-resistant* (*aux1*) mutants, have been shown to be resistant to cytokinin, ethylene, and/or abscisic acid. This suggests that the wild-type gene products may play a role in signal transduction pathways common for several plant hormones or in processes that directly affect synthesis, metabolism, or transport. The *AXR1* gene has been cloned by chromosome walking and shown to encode a protein with homology to the ubiquitin-activating enzyme E1 (Leyser et al., 1993). Although it remains to be proven that AXR1 is a functional ubiquitin-activating enzyme, the data suggest a role for protein stability in the auxin response.

Currently, only a limited number of mutants with elevated auxin levels have been isolated. JsR_1 is a tissue culture–induced duckweed mutant displaying a large frond size and a dark green color (Slovin and Cohen, 1988). Free IAA levels were elevated from two- to 100-fold, depending on the developmental

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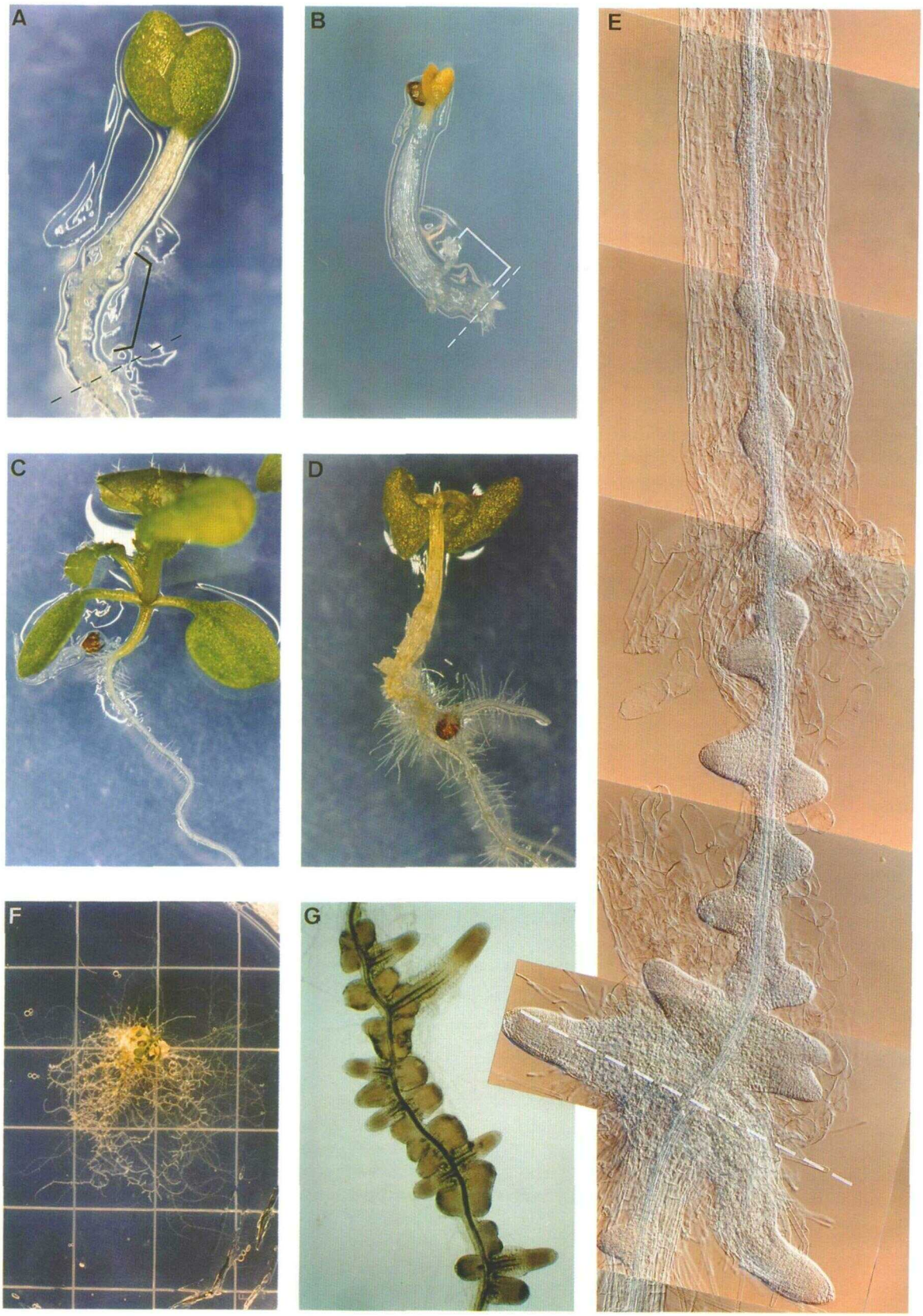

Figure 1. Phenotype of Auxin-Treated Wild-Type Seedlings and *sur1* Mutants.

stage of the plant. No auxin conjugates have been detected in this mutant. The orange pericarp (*orp*) mutant of maize is defective in tryptophan synthase (Wright et al., 1991). The level of total IAA was more than 50-fold higher, with 99% of the IAA in the conjugated form. Similarly, Arabidopsis mutants defective in tryptophan biosynthesis, *trp2-1* and *trp3-1*, have been shown to overproduce auxin conjugates and indole-3-acetonitrile (IAN). *trp2-1* also accumulates indole. Free IAA levels in these mutants are not significantly altered (Normanly et al., 1993). Although IAA has been studied since the 1930s, it has only recently been discovered from analysis of such mutants that the major route to IAA biosynthesis in duck-weed, maize, and Arabidopsis does not occur via tryptophan (Baldi et al., 1991; Wright et al., 1991; Normanly et al., 1993). It probably occurs via a compound earlier in the tryptophan biosynthesis pathway between anthranilate and indole (Normanly et al., 1993). Four nitrilase genes have been cloned from Arabidopsis (Bartel and Fink, 1994; Bartling et al., 1994). Nitrilases convert IAN into IAA. This is consistent with IAN being the direct precursor to IAA in Arabidopsis. The first plant gene involved in IAA conjugation, *iaglu*, has only recently been identified by Szerszen et al. (1994). It has been suggested that reduced or enhanced expression of *iaglu* may play a key role in the regulation of plant growth. A recent update on auxin biosynthesis and metabolism is given by Normanly et al. (1995).

In this article, we describe the isolation of Arabidopsis mutants overproducing free and conjugated IAA. These mutants are designated *superroot* (*sur1*). A detailed characterization of the mutant phenotype throughout development is presented. We investigated the phenotype of *sur1 axr2* and *sur1 constitutive triple response* (*ctr1*) double mutants, and we have obtained a map position that should permit cloning of the *SUR1* gene in the near future.

RESULTS

Isolation of *sur1* Mutants

Our interest was in isolating Arabidopsis mutants that overproduce auxin. Our strategy involved direct visual screening of mutants displaying a phenotype similar to that of auxin-treated wild-type plants. Therefore, we first analyzed the phenotype of light- and dark-grown wild-type seedlings after incubation on media containing different concentrations of 2,4-D. Depending on the applied auxin dose, different characteristics were observed. When seeds were germinated directly on medium containing 10^{-6} M 2,4-D, root outgrowth of the seedlings was completely inhibited, the cotyledons were epinastic, and the connection between the stele and the cortical and epidermal cells was broken down at the base of the hypocotyl. As shown in Figure 1A, epinastic cotyledons and hypocotyl disintegration were also observed after incubation on 10^{-5} M 2,4-D of 4-day-old wild-type seedlings. In addition, adventitious and lateral roots developed at a high frequency at the base of the hypocotyl and in the root, respectively. Figure 1B shows that the most apparent phenotypic characteristics of wild-type seedlings germinated for 6 days in the dark on medium containing 10^{-6} M 2,4-D were the absence of the apical hook, a constriction of the apical part of the hypocotyl, and the disintegration of the hypocotyl base.

To isolate the mutants, 1961 individually harvested M_2 seed stocks, derived from three independent ethyl methanesulfonate (EMS)-mutagenized populations, were scored for a phenotype similar to the auxin-treated wild-type plants. As indicated in Table 1, six mutants, designated *sur1*, were identified. *sur1-1* was isolated in a screen for mutations in regulatory genes from an EMS-mutagenized seed population that was transgenic for a homozygous, single-copy T-DNA (Boerjan et al., 1992). *sur1-1*, *sur1-3*, *sur1-4*, *sur1-5*, and *sur1-6* are in the Columbia Col-0 background, and *sur1-2* is in the Wassilewskija background. A seventh mutant (*sur1-7*) with a similar phenotype was kindly provided by K. Feldmann (University of Arizona, Tucson, AZ). This mutant is in the Wassilewskija background and was isolated from a collection of Agrobacterium seed transformants, but the mutation conferring the Sur1$^-$ phenotype was not linked to a T-DNA. Figures 1C and 1D present the phenotype of a wild-type plant and *sur1-1*, respectively. The complementation analyses shown in Table 2 indicate that all seven mutants are allelic. To determine the genetic basis of the mutation, *sur1-1* to *sur1-5* were back-crossed to the wild type and the Sur1$^-$ phenotype was scored in the F_1 and F_2 generations. The data presented in Table 3 are consistent with a monogenic recessive

Figure 1. (continued).

(A) Wild-type seedling grown for 4 days on medium without auxin supplementation and for 5 days on medium supplemented with 10^{-5} M 2,4-D. Note the epinastic cotyledons that cover part of the hypocotyl. The bracket represents the region of the hypocytol in which disintegration has occurred. The dashed line indicates the hypocotyl–root junction.

(B) Wild-type seedling germinated for 6 days in the dark on medium supplemented with 10^{-6} M 2,4-D. Note the absence of the apical hook and the constricted apical end of the hypocotyl. The bracket indicates a zone of disintegration from which hypocotyl tissue has peeled. The dashed line indicates the hypocotyl–root junction.

(C) Wild-type plant 14 days after germination.

(D) *sur1* mutant 14 days after germination.

(E) Part of the hypocotyl and the root of a 6-day-old *sur1* seedling viewed by differential interference contrast optics. The dashed line indicates the hypocotyl–root junction.

(F) *sur1* mutant 45 days after germination.

(G) A root segment of a 30-day-old *sur1* seedling viewed by light microscopy.

Table 1. Recovery of *sur1* Mutants

Seed Stock	Number of M_2 Families	Name
M_2 Col-0-1[a]	800	*sur1-1*
M_2 WS-1[b]	488	*sur1-2*
M_2 Col-0-2[c]	673	*sur1-3*
		sur1-4
		sur1-5
		sur1-6
N2370[d]	—[e]	*sur1-7*

[a] EMS-mutagenized seed stock of the Columbia Col-0 ecotype that is homozygous for the T-DNA construct described by Boerjan et al. (1992).
[b] EMS-mutagenized seed stock of the Wassilewskija ecotype.
[c] EMS-mutagenized seed stock of the Columbia Col-0 ecotype.
[d] Seed stock of the Wassilewskija ecotype, transformed by the Agrobacterium seed transformation method described by Feldmann and Marks (1987) (see Methods).
[e] Not relevant.

trait conferring the Sur1$^-$ phenotype. For convenience, only homozygous *sur1* mutants are designated *sur1* throughout the text. Heterozygous *sur1* mutants (having a wild-type appearance) are referred to as heterozygous *sur1* mutants.

Description of the Sur1$^-$ Phenotype

Seeds of heterozygous *sur1-1* mutants were surface sterilized and germinated on sterile medium in the light. Up to 4 days after germination (dag), all seedlings showed the same wild-type morphology. As indicated in Table 4, from 5 to 9 dag, the hypocotyl of the *sur1* mutant (segregating one in four seedlings) elongated on average up to twice the length of a wild-type hypocotyl and appeared less intensely green. As determined by cell number and cell length calculations, the increase in hypocotyl length was due to cell elongation rather than to increased cell number. The width of the epidermal cells was similar to that of the wild type (Table 4). The cotyledons were epinastic and developed to only approximately one-third the size of wild-type cotyledons. Up to 9 dag, the outward appearance of the *sur1* mutant root was wild type in morphology. Figure 1E shows that from 6 dag, the connection between the stele and the cortical and epidermal cells at the base of the hypocotyl disintegrated. Concomitantly, adventitious root primordia developed in this region. Neither disintegration nor adventitious root primordia were observed in 6-day-old wild-type seedlings (data not shown). In a few *sur1* mutant seedlings, hypocotyl disintegration was observed in regions where no division of the pericycle could be detected by microscopic analysis (data not shown).

Hypocotyl disintegration and the appearance of adventitious root primordia both extended in the direction of the apex. Cortical and epidermal cells either were completely separated or remained partially connected. As a consequence of a complete release of the cortex, the stele with its numerous adventitious root primordia may become fully exposed to the environment. Lateral root primordia developed at a high frequency in the root, whereas root elongation was reduced in comparison with wild-type seedlings. The density of the root hairs was, however, increased.

On average, the first leaves of the *sur1* mutant appeared at 14 dag, whereas at this stage, wild-type plants had already developed their second pair of leaves. The average *sur1* plant developed only four to six elongated and epinastic leaves, but some individuals failed to develop leaves. As shown in Figure 1F, an inflorescence is generally not produced. In some individuals, however, rudimentary inflorescences can be seen.

From ~12 dag, the morphology of individual *sur1* mutants became significantly different. Some developed high numbers of adventitious root primordia in the hypocotyl region and remained compact; others developed more elongated roots with high numbers of lateral roots.

When germinated in the dark, the disintegration of the hypocotyl and the development of adventitious roots were far less pronounced. Etiolated *sur1* mutants did not develop an apical hook, and the apical region of the hypocotyl was constricted (data not shown). In contrast to light-grown seedlings in which the frequency of adventitious root primordia was highest at the base of the hypocotyl, adventitious root primordia of etiolated seedlings developed along the hypocotyl at the same frequency. The number of adventitious root primordia was, however, lower than that in light-grown plants and could be detected only by microscopic analysis (data not shown).

A closer examination of the adventitious root development of light-grown plants showed that at the initial stages (5 dag), the cytoplasm of the pericycle cells became dense, and nuclei could easily be observed by differential interference contrast optics. In some parts of the hypocotyl, the pericycle was composed of two layers of cells. The activated pericycle was not always restricted to well-defined sites but ran throughout the hypocotyl as an uninterrupted file ascending toward the apex. The adventitious roots are initiated in pericycle cells opposite to the xylem poles, as previously described for wild-type roots

Table 2. Complementation Test of *sur1* Mutants

Cross (♀ × ♂)[a]	Sur1$^-$	Sur1$^+$	Chi Square[b]
SUR1-2/sur1-2 × *SUR1-1/sur1-1*	8	21	0.096
SUR1-2/sur1-2 × *SUR1-3/sur1-3*	4	19	0.710
SUR1-4/sur1-4 × *SUR1-2/sur1-2*	4	14	0.074
SUR1-5/sur1-5 × *SUR1-3/sur1-3*	17	71	1.515
SUR1-3/sur1-3 × *SUR1-5/sur1-5*	28	72	0.480
SUR1-6/sur1-6 × *SUR1-3/sur1-3*	10	31	0.008
SUR1-2/sur1-2 × *SUR1-7/sur1-7*	10	27	0.081

[a] All plants were heterozygous for *sur1*.
[b] Calculated values were based on an expected ratio of three wild-type plants to one mutant; $P > 0.05$.

Table 3. Genetic Segregation of Lines *sur1-1*, *sur1-2*, *sur1-3*, *sur1-4*, and *sur1-5*

Cross	Generation	Sur1⁻	Sur1⁺	Chi Square[a]
SUR1-1/sur1-1 × wild type	F_1	0	10	
	F_2[b]	37	106	0.058
SUR1-2/sur1-2 × wild type	F_1	0	11	
	F_2[b]	71	258	2.052
SUR1-3/sur1-3 × wild type	F_1	0	14	
	F_2[b]	17	59	0.281
SUR1-4/sur1-4 × wild type	F_1	0	12	
	F_2[b]	27	82	0.003
SUR1-5/sur1-5 × wild type	F_1	0	32	
	F_2[b]	49	152	0.041

[a] Calculated values were based on an expected ratio of three wild-type plants to one mutant seedling; $P > 0.05$.
[b] Approximately half of the F_2 plants segregated for the Sur1⁻ phenotype in their progeny. An example is given for one such plant.

(Steeves and Sussex, 1989). Because the Arabidopsis hypocotyl has two xylem poles, adventitious roots appeared in two distinct rows. At later stages of development, when disintegration and adventitious root formation had gradually intensified, lateral roots developed on previously developed adventitious roots, resulting in the development of roots in all directions.

A microscopic examination of individual lateral roots emerging from the main root demonstrated that they do not emerge equidistant from each other. Clusters of lateral root primordia may appear in patches, interspaced by root tissue displaying a wild-type appearance. Moreover, as shown in Figure 1G, the morphology of individual lateral root primordia is highly variable. Root primordia may grow out in a similar way as wild-type lateral roots or may stay quiescent. The individual lateral roots were often thicker compared with wild-type lateral roots. Fused primordia were frequently observed. The lateral root primordia of *sur1* mutants can develop very close to each other and very close to root tips (Figure 1G). Figure 2A shows that roots of mutants display a gravitropic response not significantly different from that of wild-type plants.

Auxin Autonomous Growth

Because auxins promote the initiation of lateral root primordia (Torrey, 1976; Wightman et al., 1980; Blakesley et al., 1991) and are required for proliferation of cultured plant cells in vitro, we investigated whether *sur1* explants could proliferate in the absence of exogenously applied phytohormones. Figure 2B shows that root explants of 18-day-old *sur1* mutants were able to propagate on medium lacking phytohormones and developed a rooty mass, whereas explants of wild-type seedlings did not develop further. Hypocotyl and cotyledon explants of *sur1* mutants were also capable of undergoing phytohormone-free propagation. Upon closer examination, we observed that these three types of cultured explants developed numerous root primordia (data not shown). This experiment demonstrates that all three organs tested (root, hypocotyl, and cotyledon) were able to sustain auxin-autonomous growth. As shown in Figure 2C, these explants differentiated into chloroplast-containing cells that subsequently gave rise to shoots when incubated on cytokinin-containing medium. In an ordinary Arabidopsis regeneration experiment, green tissue and shoots can be obtained only from wild-type root explants when incubated on auxin-containing medium prior to cytokinin-containing medium (Valvekens et al., 1988). When the regenerated *sur1* mutant shoots were transferred to medium lacking cytokinins, an inflorescence with infertile flowers developed. After prolonged incubation of these shoots on hormone-free medium, excess rooting appeared again and adventitious roots developed on the stem (data not shown).

IAA Quantification

To determine whether the endogenous auxin levels were elevated in the mutant, gas chromatography, single-ion-monitoring mass spectrometry (GC-SIM-MS) analyses were performed as described in Methods. The heterozygous *sur1-1* seed stock was germinated on hormone-free medium, and seedlings with Sur1⁻ and Sur1⁺ phenotypes were harvested separately for 5 successive days. No measurements were made for seedlings

Table 4. Characterization of Hypocotyl Cells of the *sur1* Mutant

Parameters	Age (dag)[a]	Sur1⁺	Sur1⁻
Hypocotyl length[b]	6	1.49 ± 0.18 mm	2.85 ± 0.41 mm
Hypocotyl length[b]	9	1.98 ± 0.25 mm	4.01 ± 0.55 mm
Hypocotyl cell number[c]	6	17.2 ± 1.6	16.8 ± 3.3
Hypocotyl cell length[d]	10	189.1 ± 63.5 μm	361.9 ± 106.0 μm
Hypocotyl cell width[d]	10	31.1 ± 4.5 μm	31.2 ± 4.9 μm

Measurements were performed as described in Methods. For each series of measurements, 50 individual plants were analyzed.
[a] dag, days after germination.
[b] Measured from the transition zone to the base of the cotyledons.
[c] Represents the average number of epidermal hypocotyl cells along individual cell files.
[d] Only epidermal cells are considered.

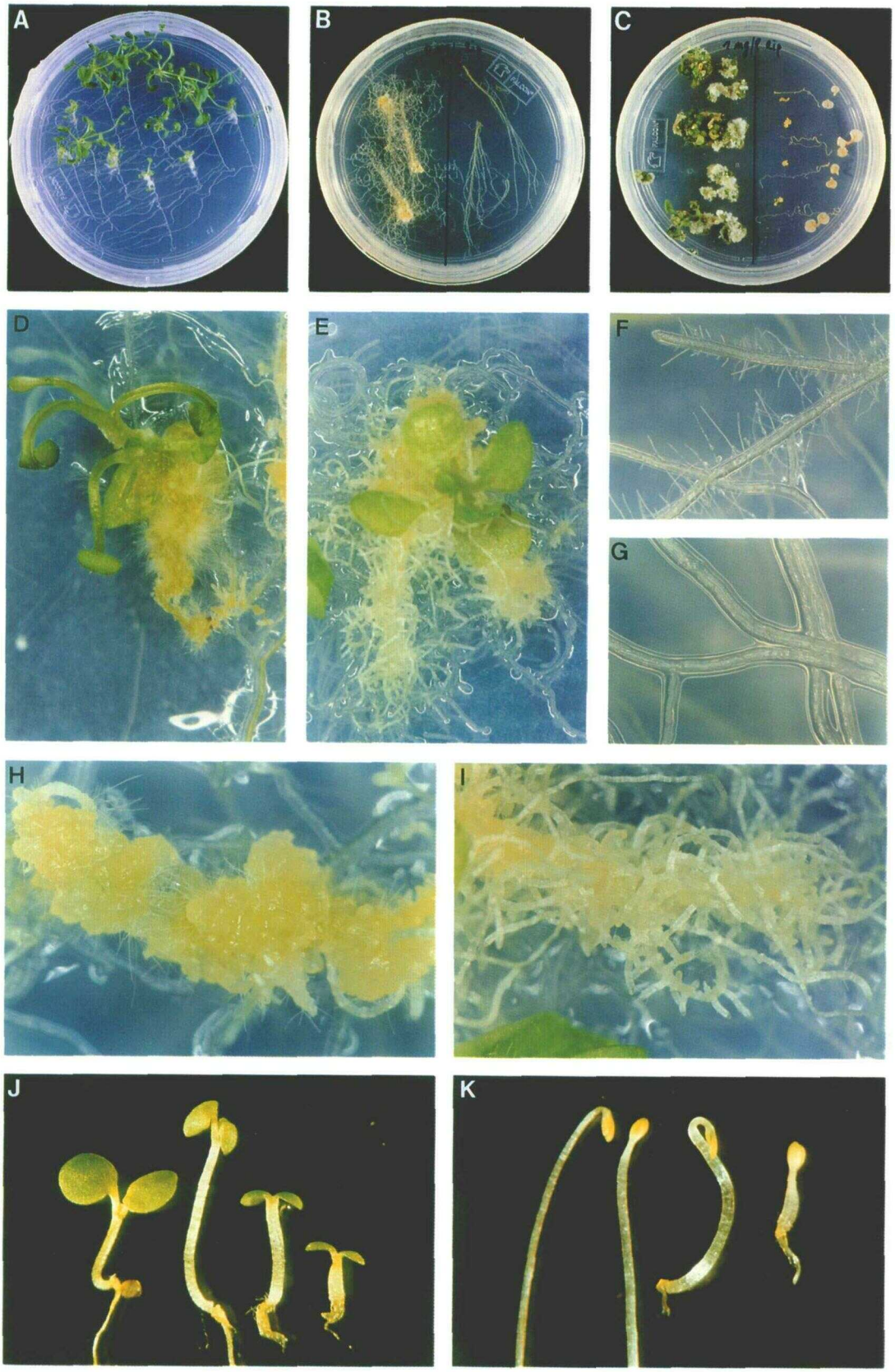

Figure 2. Gravitropic Response, Auxin-Autonomous Growth, and Morphological Analysis of *sur1 axr2* and *sur1 ctr1* Double Mutants.

Table 5. GC-SIM-MS Quantification of Free and Conjugated IAA

	Free IAA			Bound IAA			Total IAA		
Dag[a]	Sur1$^+$	Sur1$^-$	Fold Increase	Sur1$^+$	Sur1$^-$	Fold Increase	Sur1$^+$	Sur1$^-$	Fold Increase
5	1,549	2,307	1.5	92,682	76,408	—[b]	94,231	78,715	—[b]
6	5,774	13,573	2.4	20,041	58,361	2.9	25,815	71,934	2.8
7	3,711	12,113	3.3	13,138	17,334	1.3	16,849	29,447	1.7
8	86	322	3.7	3,469	22,326	6.4	3,555	22,648	6.4
9	291	643	2.2	7,320	53,784	7.3	7,611	54,427	7.2

IAA levels are expressed as picomoles per gram fresh weight of tissue. Analyses were performed on Sur1$^-$ and Sur1$^+$ progeny of a single Sur1$^+$ plant, as described in Methods. Each sample analyzed consisted of ~40 seedlings.

[a] Dag, days after germination.

[b] No increase.

before 5 dag, because the Sur1$^-$ phenotype could be clearly discriminated only from 5 dag. As shown in Table 5, in the plants with the Sur1$^-$ phenotype, the levels of free and conjugated IAA were elevated up to four- and sevenfold, respectively. Moreover, the free and conjugated IAA levels showed kinetics for Sur1$^-$ plants that were similar to those for Sur1$^+$ plants; between 5 and 6 dag, a sharp increase in the level of free IAA was observed. Between 7 and 8 dag, the free IAA pool decreased dramatically and increased again between 8 and 9 dag. The level of conjugated IAA decreased between 5 and 7 dag and increased between 8 and 9 dag.

It has been reported by Normanly et al. (1993) that IAN can be converted to IAA during hydrolysis of the IAA conjugates. To investigate the possibility that nonenzymatic hydrolysis of IAN could be the cause of the increased amount of IAA detected in the *sur1* mutant, the stable isotope [$^{13}C_1$]IAN was added as an internal standard before the hydrolysis. We analyzed two pools of 6-day-old wild-type plants and one pool of 6-day-old *sur1* mutants in this way (see Methods). Total IAA levels in the two wild-type pools were 18,520 pmol g^{-1} fresh weight of tissue with an additional 14.72% of [$^{13}C_1$]IAA and 18,644 pmol g^{-1} fresh weight of tissue with an additional 15.21% of [$^{13}C_1$]IAA, respectively. The total IAA content of the mutant pool was 70,022 pmol g^{-1} fresh weight of tissue with an additional 15.47% of [$^{13}C_1$]IAA. Because 14.67% of [$^{13}C_1$]IAA occurs naturally, these data demonstrate that the 3.8-fold increase in total IAA in the 6-day-old mutant seedlings was not due to nonenzymatic conversion of the IAN pool.

Furthermore, using an ELISA technique, we quantified the levels of free IAA, abscisic acid, zeatin, and isopentenyl adenosine from 7-day-old wild-type and *sur1-2* plants. The average level of five replicate measurements of free IAA in the wild type was 0.689 ± 0.095 pmol mg^{-1} dry weight of tissue, whereas that of *sur1-2* was 9.800 ± 0.650 pmol mg^{-1} dry weight of tissue, indicating a 14-fold higher free IAA content in 7-day-old *sur1-2* seedlings as compared with the wild type. The levels of abscisic acid, zeatin, and isopentenyl adenosine in the wild-type plants were not significantly different from those measured in *sur1-2* mutants (data not shown), suggesting that the increase in IAA content is not merely due to the morphological difference between *sur1* and wild-type seedlings.

Analysis of *sur1 axr2* Double Mutants

axr2 is a dominant auxin-resistant Arabidopsis mutant (Wilson et al., 1990). *axr2* mutants are dwarfs, do not develop root hairs, and are defective in both root and shoot gravitropism. When

Figure 2. (continued).

(A) Analysis of gravitropic response of seedlings derived from a seed stock heterozygous for *sur1*. Wild-type plants (top) and *sur1* mutants (bottom) show a similar gravitropic response.

(B) Root explants of 18-day-old *sur1* (left) and wild-type (right) seedlings after incubation on medium without exogenously supplied phytohormones for 2 more weeks. *sur1* roots have propagated and have developed root masses, whereas wild-type root explants did not develop further.

(C) Root, hypocotyl, and cotyledon explants of 7-day-old *sur1* (left) and wild-type (right) seedlings after incubation on 5 × 10^{-6} M isopentenyl adenine for 20 days. All *sur1* explants have developed green calli with adventitious roots and shoots, whereas wild-type explants died.

(D) *sur1* seedling grown for 28 days in the light.

(E) Homozygous *sur1 axr2* double mutant grown for 28 days in the light. Note the round-shaped leaves.

(F) Close-up of a root segment of a 28-day-old wild-type seedling.

(G) Close-up of a root segment of a 28-day-old *axr2* seedling indicating the absence of root hairs.

(H) Close-up of a root segment of a 28-day-old *sur1* seedling.

(I) Close-up of a root segment of a 28-day-old homozygous *sur1 axr2* seedling.

(J) From left to right, wild-type, *sur1*, *ctr1*, and *sur1 ctr1* seedlings that were grown for 7 days in the light.

(K) From left to right, wild-type, *sur1*, *ctr1*, and *sur1 ctr1* seedlings that were grown for 7 days in the dark.

Table 6. Phenotypic Characteristics of *sur1*, *axr2*, and Double Mutants

Parameters	*sur1*	*axr2*	Double Mutant
Light			
Cotyledons	E	Ex	Ex
Hypocotyl length	+	−	−
Adventitious root primordia	+ +	0	+
Disintegration	+ +	0	0
Lateral root primordia	+	0	+ +
Root hairs	+ +	0	+
Root gravitropic response	N	Ab	Ab
Dark			
Hypocotyl length	−	− −	− −
Apical hook	0	0	0
Adventitious root primordia	+	0	+
Disintegration	+	0	0
Lateral root primordia	+	0	+ +
Hypocotyl gravitropic response	V	H	H

Seedlings were germinated and grown for 7 days in a 16-hr-light/8-hr-dark cycle. +, more or increased compared with wild type; −, less or reduced compared with wild type; 0, absent; E, epinastic; Ex, expanded; N, normal; Ab, abnormal; V, vertical; H, horizontal. For growth conditions, see Methods.

germinated in the dark, *axr2* mutants develop short hypocotyls that grow along the surface of the medium. Dwarfing is mainly due to severe decreases in cell elongation (Timpte et al., 1992). To investigate whether the auxin-overproducing phenotype of the *sur1* mutant could be (partially) complemented in the *sur1 axr2* double mutant, homozygous *axr2* mutants that were simultaneously heterozygous for *sur1* were identified and the phenotype of their progeny analyzed. Table 6 summarizes the observed phenotypes of the double mutants in comparison with the plants homozygous for the single mutations. Up to 7 dag in the light, double mutants developed aerial parts similar to *axr2*, including a short hypocotyl, enlarged and round-shaped cotyledons, and no hypocotyl disintegration. A microscopic examination, however, showed the development of adventitious root primordia in the hypocotyl. Surprisingly, the root of the double mutant developed significantly more lateral roots compared with *sur1*, suggesting a greater sensitivity to auxin. As shown in Figures 2D and 2E, at 15 to 20 dag, the Sur1$^-$ phenotype became dominant over the Axr2$^-$ phenotype. At this stage, double mutants developed a similar number of leaves compared with *sur1* and no inflorescence. High numbers of adventitious roots developed, splitting the hypocotyl cortex and epidermis. Figures 2F, 2G, 2H, and 2I show the morphology of wild-type, *axr2*, *sur1*, and *sur1 axr2* roots, respectively. Lateral roots of *sur1 axr2* double mutants grew farther than did the roots of the *sur1* mutant; the *sur1* roots remained compact and were often fused (Figures 2H and 2I). The number of root hairs on the roots of double mutants was higher compared with those developed on *axr2* but significantly lower compared with those on *sur1* mutants.

When germinated in the dark for 7 days, double mutants developed a short hypocotyl that grew along the surface of the medium, similar to the *axr2* hypocotyl. Moreover, disintegration was only occasionally observed, but lateral root development was more pronounced compared with *sur1* (Table 6).

The enhanced production of lateral roots in the double mutant compared with *sur1* seemed to contradict the fact that *axr2* was an auxin-resistant mutant. The expectation was that the number of lateral roots would be lower compared with *sur1*. To reinvestigate this, 4-day-old *axr2* and wild-type seedlings were incubated on medium containing 2,4-D (10^{-7}, 10^{-6}, and 10^{-5} M) for 5 days. The results are presented in Table 7. At 10^{-6} M 2,4-D, the hypocotyls of wild-type plants disintegrated at their bases, whereas in *axr2* seedlings no disintegration was observed. A microscopic examination of the hypocotyl indicated that the degree of activation of the pericycle (adventitious root formation) by exogenously supplied auxin was similar in wild-type and *axr2* seedlings. Approximately twice the number of lateral roots from the main root of wild-type plants were induced by 10^{-7} M 2,4-D than from the *axr2* root. However, microscopic examination showed that for *axr2* roots, the complete pericycle of the root was activated, whereas in wild-type roots, only discrete regions were activated, and from these regions, lateral roots emerged. At 10^{-6} M 2,4-D, both wild-type and *axr2* seedlings developed numerous lateral roots. However, similar to the observations made at 10^{-7} M, lateral roots developed in a continuous array in *axr2* roots, whereas discrete laterals were observed in wild-type roots. Similar results were obtained

Table 7. Phenotypic Characteristics of Wild-Type and *axr2* Seedlings

	Wild Type with 2,4-D			*axr2* with 2,4-D		
Character	10^{-7} M	10^{-6} M	10^{-5} M	10^{-7} M	10^{-6} M	10^{-5} M
Lateral root primordia	+	+ +	+ + +	0	C	C
Hypocotyl length	0	0	+ +	0	0	0
Cotyledons	0	0	E	0	0	0
Disintegration	0	+	+	0	0	S
Adventitious root primordia	0	+	+ +	0	+	+ +

Seedlings were germinated and grown for 4 days on medium lacking phytohormones, followed by 5 days on medium supplemented with 2,4-D in the light. +, more or increased compared with seedlings grown without auxin supplementation; 0, no significant change; C, continuous; E, epinastic; S, splitting of the hypocotyl.

Table 8. Phenotypic Characteristics of *sur1*, *ctr1*, and Double Mutants

Parameters	*sur1*	*ctr1*	Double Mutant
Light			
Cotyledons	E	Ex	E
Hypocotyl length	+ +	−	− −
Hypocotyl width	N	+	+
Adventitious root primordia	+	0	+
Disintegration	+	0	+
Root hairs	+	+	+
Root length	−	− −	− − −
Dark			
Hypocotyl length	−	− −	− − −
Hypocotyl width	N	+	+
Apical hook	0	+ +	0
Adventitious root primordia	+	0	+[a]
Disintegration	+	0	+[a]
Root length	+	− −	− − −

Seedlings were germinated and grown for 7 days in a 16-hr-light/8-hr-dark cycle. +, more or increased compared with wild type; −, less or decreased compared with wild type; 0, absent; E, epinastic; Ex, expanded; N, normal.

[a] Observed 12 dag in the dark. For growth conditions, see Methods.

when 9-day-old wild-type and *axr2* seedlings were incubated on 10^{-6} and 10^{-7} M 2,4-D for 5 days (data not shown). Thus, when incubated on auxin-containing medium, wild-type and *axr2* seedlings appeared to develop characteristics very similar to those of *sur1* and *sur1 axr2* double mutants, respectively.

Analysis of *sur1 ctr1* Double Mutants

sur1 mutants did not develop an apical hook, which might suggest that they are defective in their response to ethylene. To investigate the possibility that SUR1 is part of the ethylene signal transduction pathway, *sur1-2* was crossed to *ctr1-6* (Kieber et al., 1993; for the isolation of the *ctr1-6* allele, see Methods). The *ctr1* mutation confers a phenotype similar to that of ethylene-treated wild-type plants. This triple response consists of an exaggeration of the apical hook, an inhibition of root and hypocotyl elongation, and a radial swelling of the hypocotyl.

Single and double homozygous mutants were germinated in both light and dark conditions. Table 8 and Figure 2J show that when *sur1 ctr1* double mutants were germinated in the light for 7 days, they displayed small and epinastic cotyledons and a short and thick hypocotyl. In addition, root elongation was inhibited in the double mutant. With additional incubation, the double mutant produced numerous adventitious roots similar to those of the *sur1* mutant. Due to the short root of the double mutant, lateral root development could not be quantified. Figure 2K shows that when germinated in the dark for 7 days, *sur1 ctr1* double mutants developed a short hypocotyl, a short root, and no apical hook.

Linkage Analysis

We determined the genetic location of the *sur1* mutation using the NW100F and CS69 marker lines (see Methods). Examination of 541 F_2 plants derived from a cross between the heterozygous *sur1* (male) and NW100F resulted in one plant with a Sur1$^-$ pyrimidine-requiring (Py$^-$) phenotype from a total of 100 Sur1$^-$ plants, indicating significant linkage with the *py* gene on chromosome 2 (data not shown). As indicated in Table 9, analysis of F_3 families derived from F_2 plants with a Py$^-$ and *erecta* (Er$^-$) phenotype localized the *sur1* gene in the vicinity of the phytochrome B (*PHYB*) gene (Reed et al., 1993). The *PHYB* gene in the Landsberg *erecta* (L*er*) background contained an XhoI restriction site that is absent in the gene from ecotype Columbia (Reed et al., 1993). A cleaved amplified polymorphic sequence marker (Konieczny and Ausubel, 1993) was developed for this site to map *sur1* relative to *PHYB*. Polymerase

Table 9. Measurement of Genetic Distances between *sur1* and *py-1*, *sur1* and *er-1*, and *sur1* and *PHYB*

Cross	Scored Phenotype of F_2	Number of F_2 Plants	Scored Phenotype of F_3	Number of F_2 Plants	Genetic Distance (cM)[a]
SUR1-1/sur1-1 × NW100F	Py$^-$ Sur1$^+$	106	Py$^-$ Sur1$^-$	33	
					14.04 ± 2.66
			Py$^-$ Sur1$^+$	73	
	Er$^-$ Sur1$^+$	108	Er$^-$ Sur1$^-$	26	
					19.35 ± 3.16
			Er$^-$ Sur1$^+$	82	
SUR1-1/sur1-1 × CS69	Sur1$^-$ $PHYB_{Col/Col}$[b]	272			
					0.50 ± 0.32
	Sur1$^-$ $PHYB_{Ler/Col}$[c]	3			

[a] Genetic distances were calculated using Kosambi's (1944) mapping function; cM, centimorgan.

[b] Col/Col means that both alleles are of the Columbia type.

[c] Ler/Col means that one allele is of the Landsberg type and one of the Columbia type.

chain reaction amplifications were performed with homozygous F_2 mutants, displaying a Sur1$^-$ phenotype, derived from a cross between CS69 (*phyB-1* in L*er*) and the heterozygous *sur1-1* (male). This cross also demonstrated that *sur1* and *phyB-1* were not allelic. From 275 F_2 seedlings with a Sur1$^-$ phenotype, three recombinants, which are heterozygous for the XhoI site, could be identified. These data located the *sur1* gene on chromosome 2 at 0.5 ± 0.32 centimorgans from *PHYB*.

DISCUSSION

We isolated seven allelic Arabidopsis mutants, designated *sur1-1* to *sur1-7*, that developed small, epinastic cotyledons and a long hypocotyl from which the connection between the stele and the cortical and epidermal cells disintegrated. These mutants developed excess adventitious and lateral roots. *sur1* mutants more than 1 month old had at maximum six to eight elongated and epinastic leaves and no inflorescences. When germinated in the dark, *sur1* mutants did not develop the apical hook characteristic for etiolated wild-type seedlings and the apical end of the hypocotyl was constricted.

Several lines of evidence suggest that the observed phenotypes are due to an elevated endogenous auxin content. First, most or all of the Sur1$^-$ phenotype was phenocopied by supplementation of auxin to wild-type seedlings (Figure 1 and Table 7). Similar morphological characteristics were also observed for Arabidopsis plants transformed with bacterial auxin biosynthesis genes (see later discussion). Second, explants of the *sur1* mutant could be propagated on medium lacking phytohormones, whereas wild-type explants died under these conditions. Third, to regenerate shoots from *sur1* explants, an incubation period on auxin-containing medium could be omitted. Fourth, the levels of free as well as conjugated IAA were significantly elevated in the *sur1* mutant.

The observations that the level of free IAA in the *sur1* mutant decreases with kinetics similar to those of the wild type and that the level of conjugated IAA is elevated in the mutant suggest that the level of free IAA in the *sur1* mutant is still controlled to a large extent by conjugation. This observation is also consistent with the general belief that auxin conjugation plays an important role in controlling the level of active auxin. In plants in which bacterial auxin biosynthesis genes are expressed under the control of their own or strong heterologous promoters, an increase in both free and conjugated auxin has been detected (Klee et al., 1987; Sitbon et al., 1991, 1992, 1993; Romano et al., 1993). It is thus most probable that the increase in auxin conjugate levels in the *sur1* mutant is a consequence of elevated free auxin levels. Interestingly, mutants with defects in tryptophan synthase also accumulate IAA: in the *orp* mutant of maize, total IAA levels increase more than 50-fold, with 99% of the IAA in the conjugated form (Wright et al., 1991). Arabidopsis *trp2-1* and *trp3-1* mutants also accumulate conjugated IAA, but free IAA levels are comparable to wild-type levels (Normanly et al., 1993). Similarly, in the *sur1* mutant, 59 to 99% of the IAA is in the conjugated form, depending on the developmental stage.

Alternatively, the increase in the auxin conjugate level might be the direct result of an enhanced activity of the auxin conjugation system. In transgenic tobacco plants producing an IAA–lysine synthetase, an increase in auxin conjugates was observed. This increase in the level of conjugates was not always correlated with a comparable decrease in the level of free IAA, indicating that de novo IAA biosynthesis is partially controlled by the level of conjugation (Romano et al., 1991; Spena et al., 1991). Although an increase in IAA conjugation is not necessarily correlated with a decrease in free IAA, significantly increased IAA levels have not been observed in these transgenic plants. Therefore, this hypothesis appears less likely.

It is noteworthy that the Sur1$^-$ phenotype resembles the hairy root disease phenotype displayed by a range of plants after transformation with the *Agrobacterium rhizogenes* Ri T-DNA. Transformation of tobacco with the root locus B gene of *A. rhizogenes* (*rolB*) under the control of the cauliflower mosaic virus 35S promoter (P35S-*rolB*) results in the development of numerous roots (Cardarelli et al., 1987). Protoplasts derived from such tobacco plants are 10^5-fold more sensitive to auxin than protoplasts of wild-type plants (Maurel et al., 1994). However, neither free nor conjugated IAA levels were modified in P35S-*rolB*–transformed tobacco plants or protoplasts (Nilsson et al., 1993; Schmülling et al., 1993; Delbarre et al., 1994). It is therefore unlikely that the *sur1* mutant is affected in its ability to sense auxin.

It is interesting to compare the phenotypic characteristics of the *sur1* mutant with those of Arabidopsis plants that have been transformed with bacterial auxin biosynthesis genes. Romano et al. (1993, 1995) transformed Arabidopsis with a P19S-*iaaM* construct (the *iaaM* gene product converts tryptophan into indoleacetamide [IAM]). Interestingly, during the isolation of the regenerants, root tissue that failed to form leaves or flowers was frequently observed and only five transformants yielding seeds could be obtained. Only two of these lines showed phenotypic effects, namely, an elongation of the hypocotyl up to twice the length of a wild-type hypocotyl, leaf epinasty, and increased apical dominance. On the average, these plants had a fourfold increase of free IAA. These phenotypes only partially correspond to those of *sur1* mutants, probably because transgenic plants with severe phenotypes could not be obtained during the regeneration procedure. Likewise, it was not possible to obtain seed from homozygous *sur1* mutants even though the development of inflorescences could be obtained in tissue culture. In addition, differences in the tissue specificity and the timing of auxin overproduction in the *sur1* mutant versus the P19S-*iaaM* transformed plants could result in the different phenotypes.

A stronger phenotype of auxin overproduction was obtained by Karlin-Neumann et al. (1991). In this case, Arabidopsis plants transgenic for a Pcab-*iaaH* construct (the *iaaH* gene product converts IAM into IAA) were isolated. These transgenic plants

are wild type in appearance, but upon incubation on medium supplemented with IAM in the light, hypocotyl disintegration was observed.

Whatever the primary cause, the *sur1* mutant overproduces IAA, and most of the characteristic phenotypes of the *sur1* mutant can be explained as being a consequence of an elevated IAA level. Sánchez-Bravo et al. (1992) showed that the auxin polar transport in basal zones of the lupin hypocotyl is slower and that these zones receive lower amounts of transported IAA than the apical, elongating regions. Suttle (1991) has demonstrated for sunflower hypocotyls that this decrease in auxin polar transport is due to a loss of function of the IAA efflux carrier with increased age. As discussed by Kaldeway (1984) and Sánchez-Bravo et al. (1992), the existence of a decrease in basipetal IAA transport might produce the so-called barrier effect. This would imply that if the amount of IAA to be transported from the apical part of the hypocotyl to its base were higher than the capacity to transport IAA, IAA would accumulate in the lower regions of the hypocotyl, where it would exert its effects. Furthermore, the same authors have demonstrated that (1) there is no noticeable basipetal movement of IAA in the cortical and epidermal tissues in which the growth-sensitive cells are localized; (2) IAA transported into the stele diffuses sideways into the cortex and epidermis; and (3) the lateral IAA migration from the stele to the outer tissues (cortex and epidermis) depends on the concentration of IAA in the stele (Sánchez-Bravo et al., 1986, 1991; Ortuño et al., 1990).

Assuming a similar situation in the Arabidopsis hypocotyl, these observations might explain the increase in hypocotyl elongation, hypocotyl disintegration, adventitious root development, and the migration of the disintegration–adventitious root formation process in the direction of the apex in *sur1*. It is conceivable that elevated endogenous IAA production results in an accumulation and spillover of IAA from the transport system into the cortex and epidermis. This spillover would first occur in the basal part of the hypocotyl as a consequence of the barrier effect. It has been shown that different concentrations of IAA exert different biological effects (Trewavas and Cleland, 1983). Hence, disintegration and adventitious root formation at the base of the hypocotyl could be the result of an increased concentration of, and/or a longer exposure to, IAA in these cells.

The migration of the disintegration–adventitious root development process in the apical direction might be explained by the fact that the activity of the IAA efflux carrier decreases with age (Suttle, 1991). As with wild-type lupin hypocotyls, the region where the highest IAA levels are detected are localized in more apical regions with increasing age (Sánchez-Bravo et al., 1992). Alternatively, the disintegration process itself might have an impact on auxin transport capacity. It is unlikely that *sur1* is defective in the polar auxin transport itself. First, the *pin-formed* (*pin1*) mutant, which is defective in the polar auxin transport, has a different phenotype compared with that of *sur1* and has reduced IAA levels (Okada et al., 1991). Second, the addition of 2,3,5-triiodobenzoic acid to the culture medium does not confer a Sur1$^-$ phenotype to wild-type plants (W. Boerjan, unpublished results).

Both disintegration and adventitious root development are independent rather than disintegration being a consequence of the disruptive forces generated by adventitious root development. This was suggested by the observation that the hypocotyls of some etiolated *sur1* mutants started to disintegrate when no sign of adventitious root primordium development was noticeable by microscopic analysis.

axr2 mutants are resistant to exogenous auxin. Because *axr2* mutants are also resistant to ethylene and abscisic acid, it was proposed that the *AXR2* gene product might have a general function in signal transduction (Wilson et al., 1990; Timpte et al., 1992). Furthermore, because the expression of the small auxin-up RNA (*SAUR*) genes is almost completely inhibited in *axr2* mutants, whereas these genes are expressed within 2.5 to 5 min after exposure to auxin in wild-type plants, the *AXR2* gene is likely to play a role very early in signal transduction (McClure and Guilfoyle, 1987; Gil et al., 1994; Timpte et al., 1994). In this article, we provide evidence that the phenotypic effects of the *sur1* mutation are the result of elevated IAA levels. Consequently, it might be expected that the phenotype of the double *sur1 axr2* mutant would be additive and depend on the local concentration of auxin in combination with the local sensitivity of the tissue to auxin.

sur1 axr2 double mutants displayed characteristics of both individual *axr2* and *sur1* homozygous mutants. At early stages of development, up to 7 dag in the light, the aerial parts of the double mutant were of the *axr2* type, whereas the increased lateral root development was even more pronounced in the double *sur1 axr2* mutant when compared with that of the *sur1* mutant (Table 6). These effects were most apparent in 6-day-old etiolated seedlings; whereas the double *sur1 axr2* mutants developed high numbers of lateral and adventitious roots, this effect was not yet observed in etiolated *sur1* mutants at this stage. Roots of the double mutant developed, paradoxically, a phenotype as if they were more sensitive to auxin.

Therefore, we compared the effect of auxin supplementation to *axr2* and wild-type seedlings. Root elongation of wild-type seedlings was inhibited at a lower concentration of auxin than that of *axr2* seedlings (data not shown). These results confirm those described by Wilson et al. (1990). Moreover, lateral roots of 28-day-old *sur1 axr2* double mutants are much more elongated than 28-day-old *sur1* mutants (compare Figures 2H and 2I). This observation is in agreement with the fact that high auxin concentrations inhibited elongation of lateral roots. *axr2* mutants appear to be more resistant to auxin when compared with the wild type with respect to root elongation. However, two observations suggest that the pericycle of both the root and the hypocotyl is at least as sensitive to auxin as that of a wild-type plant. First, *axr2* seedlings developed more lateral root primordia than did wild-type seedlings when grown on medium without auxin supplementation (data not shown); and second, when 4- or 9-day-old seedlings were incubated on auxin-containing medium for 5 days, the pericycle of *axr2*

seedlings was almost entirely activated (dividing), whereas that of wild-type seedlings was activated only in distinct areas in which lateral primordia developed. Moreover, although the hypocotyl of the *sur1 axr2* double mutant appeared to be similar to that of *axr2* at 10^{-5} M 2,4-D whereas *sur1* developed a long hypocotyl at this concentration, a similar degree of activation of the pericycle was observed in the hypocotyls of both *sur1 axr2* and *sur1* mutants.

The data presented in Table 6 suggest that the *axr2* mutation partially suppresses the Sur1$^-$ phenotype for all characteristics that can be attributed to cell elongation, that is, leaf and cotyledon epinasty, hypocotyl elongation, cell disintegration, and gravitropic response. The *axr2* mutation is additive with all characteristics that can be attributed to the pericycle, that is, lateral and adventitious root development. This is consistent with the apparent hypersensitivity of the pericycle toward auxin: *axr2* mutants develop more lateral roots compared with wild-type seedlings when germinated on medium without auxin supplementation, and the pericycle of *axr2* seedlings is activated to a greater extent than that of wild-type seedlings upon incubation on auxin-containing medium.

These results suggest that the *axr2* mutation can both increase and decrease auxin sensitivity, depending on the tissue. This strengthens the hypothesis that the wild-type *AXR2* gene has a regulatory function.

sur1 ctr1 double mutants showed characteristics of both *sur1* and *ctr1* plants. This indicates an additive effect of the two mutations, suggesting that they are unlikely to contribute in a sequential order to morphogenesis. However, the epistasy of the short hypocotyl of *ctr1* over the long hypocotyl of *sur1* suggests that the inhibition of elongation mediated by ethylene acts downstream from the elongation-promoting effect of auxin.

It is possible that the *SUR1* gene encodes a regulator of auxin biosynthesis in Arabidopsis. It will therefore be critical to clone the *SUR1* gene. Currently, we are in the process of identifying yeast artificial chromosome clones prior to complementation of the *sur1* mutant.

METHODS

Ethyl Methanesulfonate Mutagenesis

The *superroot 1-1* (*sur1-1*) mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis thaliana* ecotype Columbia Col-0 seed stock that was homozygous for the single-copy T-DNA construct described by Boerjan et al. (1992). *sur1-2*, *sur1-3*, *sur1-4*, *sur1-5*, and *sur1-6* were isolated from Arabidopsis ecotypes Wassilewskija and Columbia seed stocks that were EMS mutagenized essentially as described by Chory et al. (1989). Seeds were soaked in 0.3% (v/v) EMS for 15 hr, followed by extensive washing with water over a period of 2 to 4 hr. These M_1 seeds were sown, and the resulting M_2 seeds were individually harvested and screened for the Sur1$^-$ phenotype. Plants heterozygous for the mutation were self-fertilized, and the transmission of the phenotype was confirmed in the M_3 generation. *sur1-7* was kindly provided by K. Feldmann (University of Arizona, Tucson, AZ) and corresponds to line 186 of Stock Number N2370 of the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

The *constitutive triple response* mutant (*ctr1-6*) was identified in a T-DNA–transformed collection generated in the Laboratoire de Biologie Cellulaire et Moléculaire (Versailles, France). This mutation was shown to be an allele of the *ctr1* mutant described by Kieber et al. (1993) by characterization of the flanking genomic sequence of the T-DNA insertion carried by this mutant (M. Delarue, unpublished results).

Complementation and Genetic Segregation Analysis

Plants heterozygous for *sur1* were identified by germinating mature seeds in vitro and by observing the mutant phenotype 7 days after germination (dag). Plants to be used as female parents were emasculated and cross-pollinated. Progeny were harvested, vernalized, and germinated. The phenotype of the seedlings was scored 10 dag.

Isolation of *sur1 axr2* Double Mutants

sur1 axr2 double mutants were obtained by cross-pollinating homozygous *axr2* mutants with pollen of heterozygous *sur1-1* plants. The progeny of half of the F_1 plants segregated independently for the Sur1$^-$ and the Axr2$^-$ phenotypes, because both mutations are located on different chromosomes. Among these seedlings, mutants with a wild-type, a Sur1$^-$, an Axr2$^-$, and a double mutant phenotype were observed. We chose to compare the phenotypic differences between these different classes in the progeny of a plant that was homozygous for *axr2* and heterozygous for *sur1*. Therefore, several F_2 plants with an Axr2$^-$ phenotype were allowed to set seed, and the progeny were analyzed for the Axr2$^-$ and Sur1$^-$ phenotypes. F_2 plants whose progeny segregated one in four for phenotypic characteristics of *sur1* and did not segregate into wild-type plants were determined, and their progeny were analyzed and compared with *axr2*, *sur1*, and wild-type plants.

Isolation of *sur1 ctr1* Double Mutants

sur1 ctr1 double mutants were obtained by cross-pollinating *ctr1-6* with pollen of a heterozygous *sur1-2* mutant. F_1 plants were identified that were heterozygous for both mutations by analyzing the phenotype of their progeny. The analysis of the *sur1 ctr1* double mutants was performed on the progeny of these plants.

Mapping of *sur1*

The marker lines CS69 (mutant in the gene encoding phytochrome B; *phyB-1*) and CS3077 (the *altered-auxin response* mutant *axr2*) were kindly provided by the Arabidopsis Biological Resource Center at the Ohio State University (Columbus, OH). The multiple marker line NW100F (*angustifolia-1*, *apetala-1*, *pyrimidine-requiring-1*, *erecta-1*, *long hypocotyl 2-1*, *glabra 1-1*, *brevipedicellus-1*, *eceriferum 2-2*, *transparent testa 3-1*) was a kind gift of the Nottingham Arabidopsis Stock Centre. Genetic recombination frequencies and map distances were calculated according to Koornneef and Stam (1987) and Kosambi (1944). To amplify the 840-bp promoter fragment of the phytochrome B (*PHYB*) gene (Reed et al., 1993), two oligonucleotide primers were constructed as follows: 5′-CCCAATCAATCTCTCCCTCACATTA-3′ (from nucleotide 1879 to 1903) and 5′-ACATTTCTCTGGCGTTTTCACTGTA-3′ (from

nucleotide 2695 to 2719). Plant DNA was prepared according to Dellaporta et al. (1983) and treated with RNase. The reaction mix was composed as follows: 200 ng of plant DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, 100 μM each deoxynucleotide triphosphate, 250 ng of each oligonucleotide primer in a final volume of 100 μL. Reactions were cycled automatically through time/temperature cycles as follows: one cycle of denaturation for 1 min at 94°C followed by the addition of 2 units of Taq polymerase (Beckman Instruments, Fullerton, CA). This was followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 55°C, and extension for 1 min at 72°C. To detect the polymorphism, the polymerase chain reaction product was digested with XhoI.

Morphological Characterization

For morphological comparisons, wild-type and mutant seedlings were grown in a 16-hr-light/8-hr-dark cycle at 23°C, 60% RH. For anatomical and morphometric analyses, complete seedlings were fixed overnight in 45% ethanol, 5% acetic acid, and 5% formaldehyde, followed by three washes in distilled water for 2 hr, and cleared in chloral hydrate and lactophenol for 24 hr (Beeckman and Engler, 1994). Specimens were photographed under bright-field illumination or by differential interference contrast using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). Measurements and countings were performed using a light microscope (type M12; Wild, Heerbrugg, Switzerland) equipped with a camera lucida. For the analysis of the gravitropic response, seeds of a heterozygous seed stock were germinated in the light on vertically oriented plates. After 14 days, the plates were turned over 90° and observed 10 days later.

Quantification of Endogenous Hormone Levels

For gas chromatography, single-ion–monitoring mass spectrometry (GC-SIM-MS) analyses, fresh plant material (corresponding to a pool of ~40 seedlings) was carefully weighed, frozen in liquid nitrogen, and stored at −80°C. For extraction of free and conjugated indole-3-acetic acid (IAA), the material was ground in liquid nitrogen using a mortar and pestle. After addition of 150 ng of [$^{13}C_6$]IAA (Cambridge Isotope Laboratory, Cambridge, MA) as an internal standard (Cohen et al., 1986), the material was extracted overnight in 90% methanol. After centrifugation, the supernatant was collected, and the pellet was rinsed with water for 1 hr. A second centrifugation provided a supernatant that was pooled with the methanolic extract. The volumes were such that the final extract was a 1:1 methanol/water mixture. This extract was divided into two parts.

One part was purified by solid-phase purification according to Prinsen et al. (1991). The samples were methylated by diazomethane and dried under nitrogen (Schlenk and Gellerman, 1960). The residue was dissolved in 25 μL of heptafluorobutyryl (HFB) imidazole (Chrompack International, Antwerp, Belgium) and reacted for 2 hr at 85°C in a 1-mL conical reaction vial. After chilling on ice, the reaction mixture was diluted with 400 μL of water and the IAA methyl-HFB/[$^{13}C_6$]IAA methyl-HFB was extracted twice with 400 μL of hexane. The hexane phase was washed with 400 μL of water and transferred into a small glass tube, dried under a nitrogen stream, and redissolved in 30 μL of hexane (Pilet and Saugy, 1985).

Splitless injections of 2 μL were made into a GC-SIM-MS system equipped with a silicon-encapped wall-coated open tubular fused silica capillar (SE 54 WCOT; Alltech, Gent, Belgium) of 25-m length and 0.3-mm internal diameter. A linear temperature gradient was applied from 50 to 220°C with an increase of 25°C min^{-1}. The injection temperature of the GC was 350°C, the ion source temperature of the MS was 180°C, and a helium flow of 1 mL min^{-1} was applied. The ionization potential was 70 electronvolt. For IAA quantification, the *m/z* values of the base peaks (326 for IAA methyl-HFB and 332 for [$^{13}C_6$]IAA methyl-HFB) were used. Calculations were performed according to the principle of isotope dilution.

To quantify the total amount of IAA (free and conjugated), the other part of the extract had to be hydrolyzed. After drying with a rotating vacuum concentrator (Büchi, Slawil, Switzerland) and redissolving in a smaller volume of methanol and water, the samples were hydrolyzed with 7 N NaOH at 100°C for 3 hr under a water-saturated nitrogen stream (Bialek and Cohen, 1989). Samples were desalted by passing them through a C18 column after dilution with water and adjusting the pH to 2 with 2 N HCl. IAA was eluted with diethyl ether. The samples were dried after removing the water residue and redissolved in 50% methanol. Additional purification and measurements were performed as described previously. By subtracting the concentration of free IAA from the total, the concentration of the conjugates was obtained. The experimental error was calculated taking the extraction yield and the integration error into account and was at maximum 5% of the values presented in Table 5.

To investigate the possibility of nonenzymatic conversion of indole-3-acetonitrile (IAN) to IAA, a second set of GC-SIM-MS measurements was performed. The methodology was similar to the one described previously, but in this experiment, 170 ng of solid-phase–purified [$^{13}C_1$]IAN was added before hydrolysis of the conjugated IAA. The ion that was monitored by GC-SIM-MS (IAA methyl-HFB without $COOCH_3$ with a molecular weight of 326) contains three carbon atoms and one nitrogen atom. These two elements resulted in 14.67% [$^{13}C_1$]IAA (Millard, 1978). The quantity of [$^{13}C_1$]IAN added is sufficient to discriminate the 5% conversion of IAN to IAA from the natural abundance of [$^{13}C_1$]IAA in the endogenous IAA pool.

For the ELISA tests, 600 7-day-old light-grown *sur1-2* seedlings, corresponding to ~500 mg of fresh material, were frozen in liquid nitrogen, crushed and lyophilized, extracted in nonoxidative methanol/water (80:20 [v/v]), prepurified, and HPLC fractionated using a 0.2% (v/v) formic acid/methanol gradient; the ELISA quantification of IAA and abscisic acid was performed as previously described by Julliard et al. (1992). The hormonal content was determined five times for each experiment. The same method was used for cytokinin extraction and fractionation. ELISA quantification of cytokinins was performed with polyclonal antibodies raised against zeatin riboside and against isopentenyl adenosine (Besse et al., 1992).

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